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Letters to the Editor

False-Positive Results Obtained by Following a Commonly Used Reverse Transcription-PCR Protocol for Detection of Influenza A Virus

During the course of the surveillance program for avian influenza (AI) in wild birds that is being carried out routinely at our laboratory (Centre de Recerca en Sanitat Animal, Barcelona, Spain) in the northeastern part of Spain (Catalonia), several false positives for the AI virus were detected in samples from specific wild bird species when the reverse transcription (RT)-PCR technique described by Fouchier et al. (1) was used. From January 2005 to February 2006, more than 650 samples from wild birds in this region were tested with this technique, complemented with sequencing of the positive bands obtained.

Cloacal and tracheal swabs taken from dead birds (of the species *Streptopelia decaocto* [*n* = 4], *Larus michahellis* [*n* = 2], *Vanellus vanellus* [*n* = 1], *Larus melanocephalus* [*n* = 1], *Phoenicopterus ruber* [*n* = 2], *Ardea cinerea* [*n* = 1], *Porphyrio porphyrio* [*n* = 1], and *Gallinula chloropus* [*n* = 1]) were routinely analyzed using the M52C (5′-CTTCTAACCGAGGT CGAAACG-3′) and M253R [5′-AGGGCATTTTGGACAA A(G/T)CGTCTA-3′] primers and the RT-PCR procedure described by Fouchier et al. (1). A weak band was obtained for each animal with at least one of the swabs analyzed. These bands were purified (Minielute gel extraction kit; QIAGEN), and their PCR products were sequenced (BigDye Terminator v.3.1 cycle sequencing kit; Applied Biosystems). A comparison of the sequences obtained from the GenBank database using BLASTN (NCBI) indicated a high homology (98%) of the RT-PCR products with the *Gallus gallus* clone AY006 28S rRNA gene (accession number DQ018757). The size of the DNA fragment obtained and sequenced was 235 bp and, therefore, could not be distinguished on the 2% agarose gel stained with ethidium bromide from the expected band of matrix gene segment 7 of influenza A virus (245 bp), as described in the work of Fouchier et al. (1).

A detailed study of the M52C and M253R primers and the *Gallus gallus* 28S clone AY006 rRNA sequence showed that the 3′ termini of the M52C and M253R primers shared 7 and 5 nucleotides, respectively, with the sequence of the *Gallus gallus* 28S rRNA gene (Fig. 1). Although the 28S rRNA gene sequences of the aforementioned wild bird species are unknown, this is a highly conserved gene among avian species, and therefore, it is not surprising that the primers described by Fouchier et al. (1) could amplify the 28S rRNA gene fragment in those samples. It is known that the nucleotides located at the 3′ terminus of a primer are very important for the efficiency of the polymerase extension. Consequently, the presence of 5 or 7 coincident nucleotides at this 3′ terminus could justify this unexpected amplification. In addition, all the samples, cloacal and tracheal swabs, that resulted in false positives in the RT-PCR were tested directly, without being inoculated in specific-pathogen-free eggs; thus, it is highly probable that the presence of avian cells or cell fragments in the sample could be the source of the 28S rRNA that was amplified. This unwanted amplification could not be avoided by sequentially increasing the annealing temperature from 45°C to 65°C.

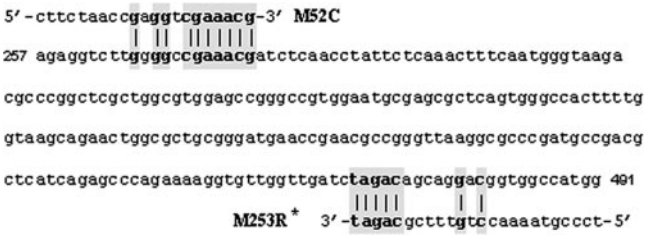

FIG. 1. Comparison of *Gallus gallus* clone AY006 28S rRNA gene and M52C and M253R oligonucleotides. Coincident nucleotides are shown in bold and in shaded boxes. *, M253R oligonucleotide reverse complementary sequence.

The increase in use of this RT-PCR procedure for testing wild bird samples for avian influenza prompted us to communicate these results.

REFERENCE

1. **Fouchier, R. A., T. M. Bestebroer, S. Herfst, L. Van Der Kemp, G. F. Rimmelzwaan, and A. D. Osterhaus.** 2000. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. J. Clin. Microbiol. **38:**4096–4101.

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